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Sensitive Analysis of Retinyl Esters by Isocratic Adsorption Chromatography

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Summary: A sensitive method for the determination of retinyl esters, including their geometric isomers, by isocratic adsorption HPLC is described. The development of a special recycling system allows the separation of all-*trans*-, 13-*cis*, 11-*cis*- and 9-*cis*-retinyl palmitate, -stearate, -oleate, -palmitoleate and -linoleate with short retention times and high sensitivity. Extraction of the retinyl esters from various organs with mobile phase avoids additional evaporation steps. The method was evaluated by the determination of retinyl esters in small tissue samples such as tongue, trachea and inner ear. This is the first report of the detection of retinyl esters in these tissues, which are known to depend on an adequate vitamin A supply. The main ester in all cases was retinyl palmitate, followed by retinyl stearate and oleate.

Introduction

Vitamin A occurs in various forms and represents a group of similar chemical compounds with different biological effects and different routes of metabolism. The predominant form of vitamin A found in biological samples is the alcohol (retinol), which occurs as an ester of long chain fatty acids. Retinyl esters are formed and stored in the liver (1), the intestine (2, 3) and in the retina (4, 5). Ninety seven percent of the vitamin A stored in the liver, which is the major storage depot, is esterified whith palmitic acid, stearic acid or oleic acid (1, 2, 6). These esters are even found in the eye (1, 7), appearing in the vision cycle, and protecting the retina from hypovitaminosis. The presence of this storage form of vitamin A in the retina demonstrates a functional dependence on the availability of vitamin A, which again may be due to a dependence on other tissues, such as the liver.

With regard to the hypothesis that retinyl esters in different extra-hepatic organs might reflect a functional significance or dependence of the particular tissue upon this vitamin, it seems appropriate to determine the retinyl esters in those tissues that are functionally dependent on an adequate vitamin A supply.

Functional dependence on the vitamin has been reported for the tracheobronchial tract (8), the testicle (9) and the tongue (10). To evaluate whether retinyl esters or their geometric isomers occur in these tissues, it is necessary to design a highly sensitive method so that even trace amounts of retinyl esters can be separated and detected. The most effective method is high performance liquid chromatography (HPLC). Different conditions for the separation of retinyl esters have been described (11–14), but the various published methods do not fulfil the special demands of trace analysis, such as short retention times with high peaks, and the special treatment of small tissue samples to avoid additional evaporation and derivatisation steps. Either retention times in a gradient system were too long for the preservation of high peaks (11, 14), or the main retinyl esters (palmitate, stearate,

oleate, palmitoleate, linoleate) were not completely separated (13), or the extraction procedure required additional steps in order to adapt it to a reverse phase system (11). Thus all these methods were not practicable for the trace analysis of retinyl esters in small tissue samples. We therefore had to develop a suitable procedure for the qualitative and quantitative analysis of different retinyl esters, especially those found in trace amounts.

Materials and Methods

Tissue samples were collected from 10 normally nourished male guinea pigs (Himalayan spotted), mean body weight 525 ± 50 g and from 4 vitamin A depleted guinea pigs (340 ± 22 g). The animals were depleted as described recently (15). Chemicals, all HPLC-grade, were obtained from the following sources: *n*-hexane, 2-propanol (Aldrich Chemical, Wisconsin USA), diisopropylether, dioxan, acetonitrile, Al_2O_3 (Merck, Darmstadt, FRG); external standards as given in table 1 were kindly supplied by Hoffmann-La Roche.

Tab. 1. Purities and impurities of external standards of different retinyl esters estimated by HPLC and derivative spectroscopy.

Standard	Purity	Impurities	
all-*trans*-retinyl palmitate	99.0%	0.3% 13-*cis*	16:0
		0.7% 9-*cis*	16:0
9-*cis*-retinyl palmitate	99.7%	0.3% all-*trans*	16:0
11-*cis*-retinyl palmitate	98.2%	0.7% all-*trans*	16:0
13-*cis*-retinyl palmitate	99.2%	0.4% all-*trans*	16:0
all-*trans*-retinyl stearate	81.5%	12.7% all-*trans*	16:0
11-*cis*-retinyl stearate	90.5%	4.0% 13-*cis*	18:0
		1.9% 9-*cis*	18:0
		2.6% all-*trans*	18:0
all-*trans*-retinyl oleate	75.5%	4.3% all-*trans*	16:0
		1.0% all-*trans*	18:0
		9.0% all-*trans*	18:2
		5.0% all-*trans*	16:1

Equipment

The HPLC system for determination of the retinyl esters consisted of a model LC-85 UV detector (Perkin Elmer) and 650-10S fluorescence detector (Perkin Elmer). A rheodyne injector (Model 7125) and a Series 1 pump (Perkin Elmer). Peak areas and peak heights were calculated using a Packard integrator type 610 (Packard, Frankfurt, FRG) and chromatograms plotted with a plotter 561 (Perkin Elmer, Frankfurt, FRG).

The column (Spherisorb Silica, 250 × 4.6 mm) selected for the separation was filled with 3 µm packing material, and purchased from Bischoff (Leonberg, FRG). Pre-columns (25 × 4.6 mm) containing the same packing material were coupled to each analytical column. As a second test column we chose a Spherisorb Silica with 5 µm packing material and shorter length (125 × 4.6 mm) purchased by Perkin Elmer (Frankfurt, FRG).

The samples were weighed on Mettler analytical balances type M5SA (± 0.025 mg) and type H35 (± 0.1 mg).

Procedures

Preparation of samples

The handling of the tissues from the dissection up to the injection of the extracted retinyl esters was carried out under dim red light.

After dissection, the tissues were quickly frozen in liquid nitrogen and freeze-dried immediately (Beta A, Christ, Osterode, FRG) at −55 °C and 3×10^{-3} Torr for 24 to 48 hours, depending on the tissue weight. The freeze-dried samples were homogenized in a mortar. Small amounts (20−100 mg) of the tissue powder were then placed in closed glass tubes and weighed. The tissue samples were mixed with *n*-hexane (1 ml), homogenized (60 s) with an Ultra Turrax (IKA-Werk, Staufen, FRG), and centrifuged (5000 min^{-1}, 2 min). The organic layer was pipetted off and transferred to a Teflon-lined brown glass tube. After freeze-drying of tissue from the inner ear (sample weight less than 0.5 mg) and preparation of the membranous cochlea, weighed samples were transferred to small glass tubes (Kontes Micropotter, Michigan, USA), each with a matching polished glass pestle. After addition of 100 µl *n*-hexane, each sample was homogenized (20 s) and centrifuged. After centrifugation the upper part of the organic layer was carefully pipetted off to avoid transfer of biological material due to the low extraction volumes and injected into the HPLC-system.

Preparation of standards

Small amounts of the retinyl ester standards (about 25 mg) were dissolved in 100 ml 2-propanol in brown glass flasks (final concentration about 250 mg/l). An aliquot of this stock solution was diluted with 2-propanol until an $A^{1g/l}_{328\,nm}$ absorbance of about 0.5 at 328 nm was measured against 2-propanol (UV/VIS 550, Perkin Elmer, Frankfurt, FRG). The concentration was calculated from this value, using the specific $A^{1g/l}_{328\,nm}$ absorbance of retinyl palmitate in ethanol (940 at 328 nm). Thus the retinyl palmitate concentration of the stock solution was about 5 mg/l. The purity of the external standards was estimated by calculating the typical minima of the derivative spectra of the compounds in question by the method described recently (16).

The spectrophotometer was calibrated by comparison of the absorbance of a given standard either with a different detector (UV double beam spectrophotometer 124, Perkin Elmer, Frankfurt, FRG) or with the dilution of an external standard (retinyl palmitate) of known weight. The resulting calibration plot was linear and could be extrapolated through the origin.

The stock solution was stable for six weeks when stored in the dark at −28 °C. Reference solutions from 10 to 100 µg/l of each retinyl ester were obtained by diluting the stock solution with *n*-hexane in small quantities. These reference solutions, however, were not quite as stable as the stock solution when kept in a refrigerator at 4 °C. The external standards have to be freshly prepared each day from the stock solution. Solutions of low concentration are especially unstable under typical laboratory conditions (light, oxygen contact and room temperature) (tab. 2).

Chromatographic conditions

The chromatographic conditions for separation of the different retinyl esters were as follows. Mobile phase: *n*-hexane: diisopropyl ether (98.5 + 1.5 by vol.), flow rate: 2.0 ml/min, detection: fluorescence-excitation 332 nm, emission 472 nm, slit 10/10 nm, and UV-detection: 325 nm, at room temperature.

In order to achieve an adequate separation of retinyl esters, the system has to be equilibrated for up to 24 hours in a closed recycling system, which pumps the pre-dried eluent (molecular sieve) over a moisture control system, as shown in figure 1.

Tab. 2. Time-dependent concentration of two different retinyl ester standards after dilution from the stock solution under different typical laboratory conditions (dark at 4 °C: refrigerator; light and room temperature: laboratory). Values represent 3 replicates at each time point.

Condition	Concentration (%) at different times after dilution (minutes)					Compound
	0	15	30	45	60	
4 °C (dark)	100	101.6 ± 3.6	103.2 ± 2.1	101.2 ± 4.3	99.2 ± 2.8	Palmitate
	100	100.7 ± 2.0	101.5 ± 3.6	103.2 ± 5.7	98.4 ± 4.1	Stearate
22 °C	100	97.5 ± 3.1	97.5 ± 4.2	94.8 ± 6.1	94.0 ± 6.9	Palmitate
	100	99.2 ± 1.9	96.1 ± 5.7	93.7 ± 5.9	92.2 ± 7.1	Stearate

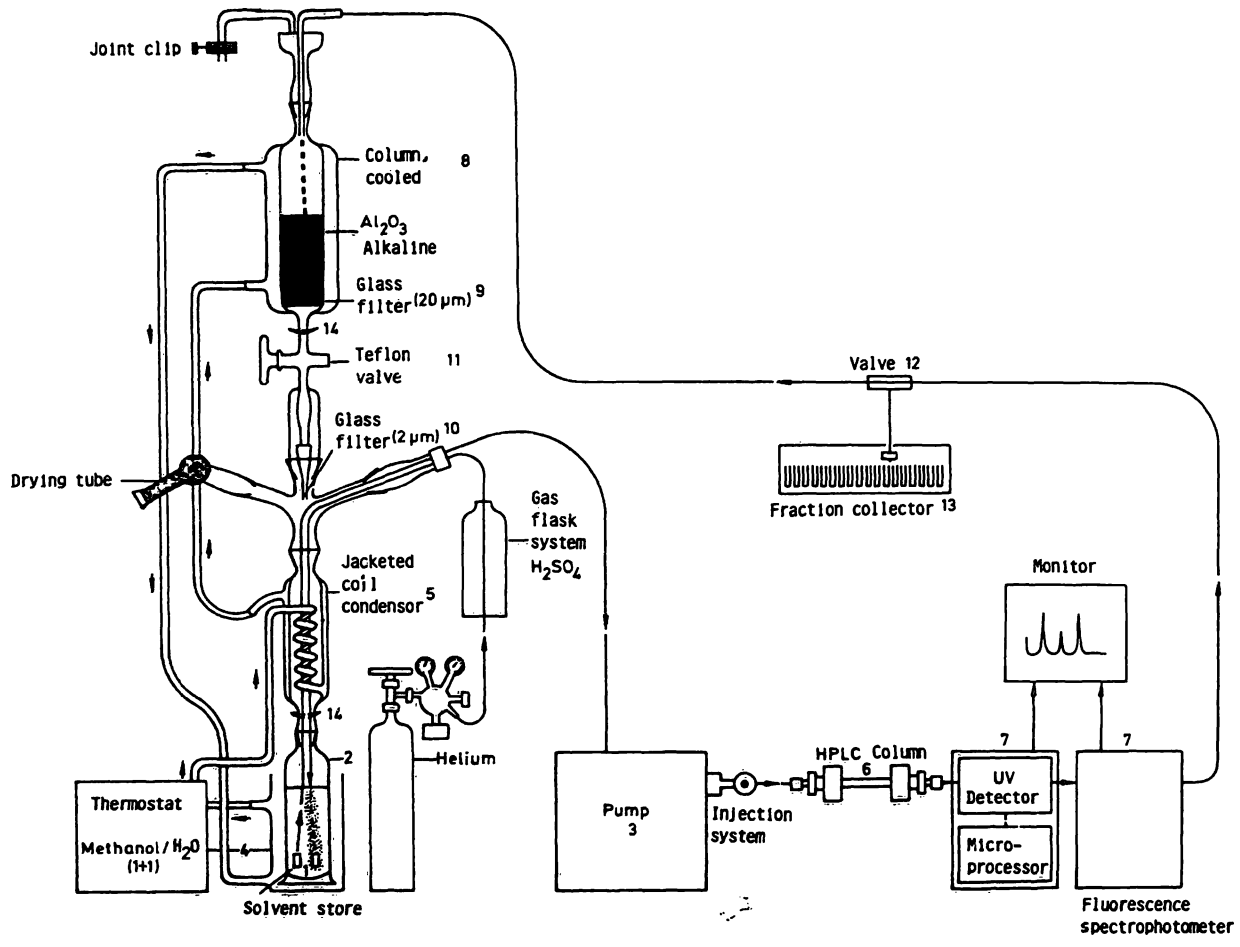

Fig. 1. Recycling- and moisture control system as discussed in the text.

After mixing, the components of the mobile phase were passed over a drying column ⟨1⟩ containing alumina in a brown glass flask ⟨2⟩, thereby removing water from the mobile phase by "adsorptive filtration". The dry solvent was degassed in an ultrasonic bath; then aspirated from the flask into the HPLC-pump ⟨3⟩. During the analysis, the mobile phase was degassed with helium, which was passed through a gas flask system containing sulphuric acid.

During the chromatographic separation process the storage flask and the recycling system were thermostatted (usually at 14 °C) ⟨4⟩. The thermostatted jacketed coil condensor ⟨5⟩ which was mounted on the storage flask prevented one or both eluents of the mobile phase from condensing. After flowing through the column ⟨6⟩ and the detector ⟨7⟩ the mobile phase reached a dropping funnel, which was connected to the thermostatted system and contained dried neutral alumina ⟨8⟩. The dropping funnel was sealed with a 20 µm fritted glass disc ⟨9⟩, to which a separate shell containing a 2 µm fritted glass disc was connected ⟨10⟩, and which could be easily separated and purified when contaminated with alumina particles. At the exit of the dropping funnel, a three-way stopcock ⟨11⟩ controlled the flow of the eluent, so that the alumina of the solid-phase was always covered with the eluent. An opening to the atmosphere was provided via a drying tube, which was filled with blue silica gel. Between the detector and the dropping funnel was a fraction collector with a three-way-valve, which permitted the collection of the separated compounds for further analysis, or served to prevent overloading of the alumina column.

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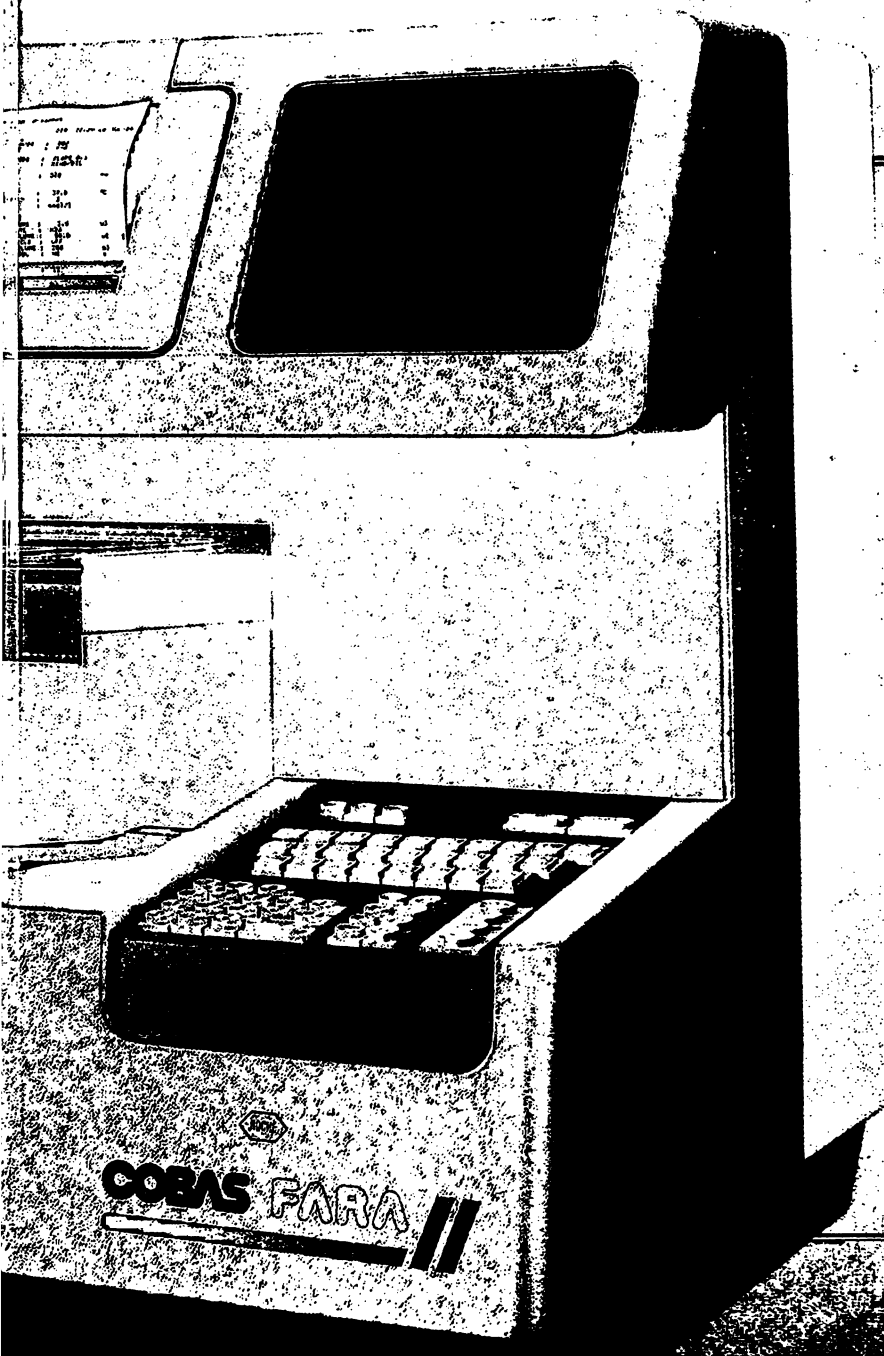

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Qualitative and quantitative analysis

The qualitative analysis of biological samples was carried out by different methods: Comparison of typical retention times of external standard and sample, recording of fluorescence and/or UV-spectra in the stop flow mode and peak purity testing by the absorbance ratio method (16). The use of two different detection modes allowed us to apply the ratio of the relative responses of the UV- and fluorescence detectors, as recommended by *Snyder & Kirkland* (17). This response ratio method is advantageous for the trace analysis of retinyl esters, because each retinyl ester isomer can be characterized by its typical ratio (see tab. 8A).

Quantitative analysis was carried out with external standards (injection volume 25, 50 and 100 µl; multiple point calibration) as given in table 1, and by comparison of peak heights as suggested by *Snyder & Kirkland* (17) for trace analysis under isocratic conditions, when stable and reproducible retention times are ensured. Internal standards, such as the unphysiological ester retinyl margarinate (C_{17}), which behaves like the physiological esters, were tested, but excluded because they showed the same retention time as retinyl palmitate (C_{16}).

Results

Extraction

The first step in the extraction procedure is to freeze dry the sample, a process which denatures proteins and cellular membranes, and therefore enables us to work without typical denaturation media such as acetonitrile or ethanol. Extraction of freeze-dried samples with acetonitrile and hexane or other solvents does not result in higher extraction values for retinyl esters (tab. 3). Freeze-drying furthermore favours the next step: homogenizing of the tissues in a mortar. Especially for tissues like liver or kidney, homogenization in a mortar overcomes the problem of inhomogenous distribution of retinyl esters within the tissue; in such cases, the whole organ is homogenized, the sample volume is large and only an aliquot is taken for estimation. When wet liver samples are homogenized in physiological NaCl, e. g. with a vortexer, the within-run precision shows greater deviations (CV 5.9%; n = 6) than the precision of freeze dried and mortared samples (CV 3.5%; n = 6).

Tab. 3. Quantitative effects of different solvents for extraction of retinyl palmitate from a freeze-dried liver sample, expressed in % peak height relative to the peak height of the retinyl palmitate peak extracted with pure *n*-hexane(taken as 100%). Each value represents the mean of 4 replicates from one powdered liver (10 mg dry weight each).

Extraction solvent	Volume ratio	% Peak height
n-hexane pure		98.7 ± 3.8
hexane : acetonitrile	2:1	96.1 ± 4.2
hexane : acetonitrile : H_2O	3:1:1	71.4 ± 11.3
hexane : acetonitrile : H_2O	1:1:1	78.7 ± 9.4
ethanol : H_2O	6:4	40.0 ± 4.1
ethanol : H_2O	9:1	84.3 ± 8.2
ether : hexane	1:1	81.6 ± 13.4

A repeat extraction of the homogenate of 5 liver samples indicated that 3% of the main retinyl ester in the sample, retinyl palmitate, had not been extracted. This is due to the fact that the extraction phase cannot be totally pipetted off from the tissue.

The recovery from "blank" samples was tested by adding known amounts of a standard mixture of retinyl esters (palmitate, stearate, oleate, palmitoleate and linoleate) to 4 liver samples (20 mg dry weight, each), 4 testicle samples (10 mg dry weight each) and 2 trachea samples (20 mg dry weight each) from two vitamin A deficient guinea pigs, followed by extraction and separation as described. The determination of retinyl esters in the tissue samples of the depleted animals, prior to the addition of standard mixtures showed that neither retinol nor retinyl esters were detectable. The recoveries for added retinyl palmitate and stearate to the blank samples varied between 92% and 106%, whereas the recoveries of added oleate, palmitoleate and linoleate ranged from 81% to 96%. The recovery and linearity of extraction of retinyl esters from biological samples from normally nourished animals is shown in table 4.

Table 5 and figure 2 demonstrate the recovery and the stability of retinyl esters with respect to isomeri-

Tab. 4. Concentration and linearity of the main tissue-related retinyl esters (palmitate 16:0; stearate 18:0 and oleate 18:1) from liver and lung homogenates.

Sample	Dry weight	Retinyl ester concentration (ng per sample weight)					
		16:0	CV	18:0	CV	18:1	CV
Liver: (6)	10 mg	1250 ± 48.7	3.9%	510 ± 20.7	4.0%	90 ± 4.3	4.7%
(6)	5 mg	670 ± 27.4	4.1%	280 ± 9.0	3.2%	51 ± 2.8	5.5%
(4)	1 mg	140 ± 8.5	6.1%	60 ± 3.3	5.5%	11 ± 0.7	6.4%
Lung: (3)	50 mg	38 ± 2.4	6.3%	6 ± 0.4	6.6%	9 ± 0.5	5.5%
(3)	25 mg	20 ± 1.6	6.8%	4 ± 0.3	7.5%	5 ± 0.4	8.0%
(3)	5 mg	4 ± 0.4	10.0%	n.d.		2 ± 0.1	10.0%

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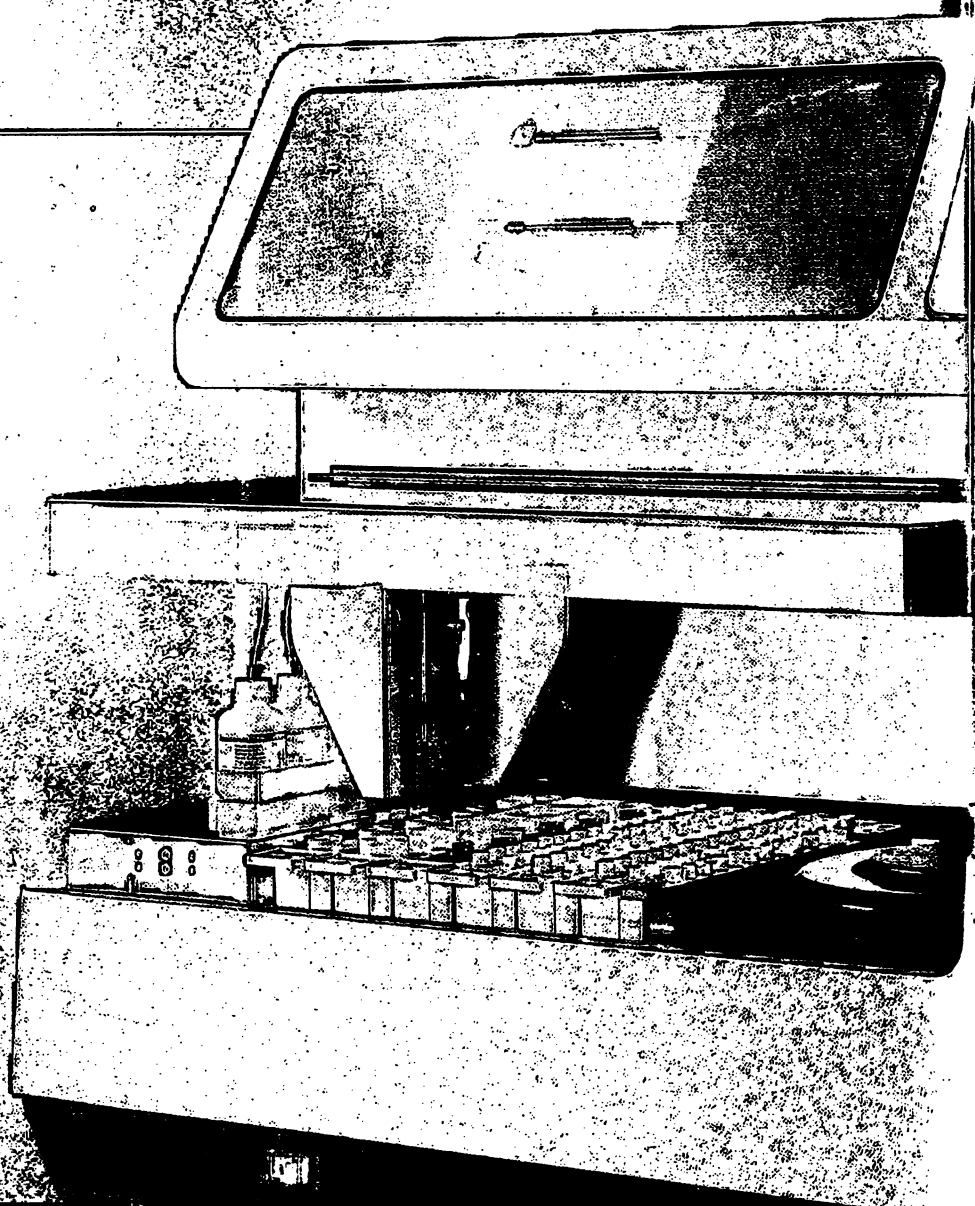

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Tab. 5. Calculated and real values of retinyl esters (expressed as ng/ml extraction volume) after the addition of a known quantity of retinyl ester standard mixture (in 1 ml *n*-hexane) to a biological sample (testicle) already containing a known concentration, and extraction in 1 ml *n*-hexane as described (injection volume 100 µl).

Compound		Sample	Standard	Mixture (1 : 1)		CV
				Calculated	Measured	
13-*cis*	18 : 0	3	8	5.5	5.5	0%
13-*cis*	16 : 0	9	35	22.0	22.5	2%
11-*cis*	18 : 0	n. d.*	8	4.0	4.5	12%
11-*cis*	16 : 0	n. d.*	8	4.0	4.5	12%
9-*cis*	16 : 0	2	7	4.5	5.0	11%
all-*trans*	18 : 0	66	60	63.0	66.0	5%
all-*trans*	16 : 0	146	98	122.0	128.0	5%
all-*trans*	18 : 1	18	22	20.0	20.5	2%
all-*trans*	16 : 1	3	2	2.5	2.5	0%
all-*trans*	18 : 2	8	3	5.5	5.5	0%

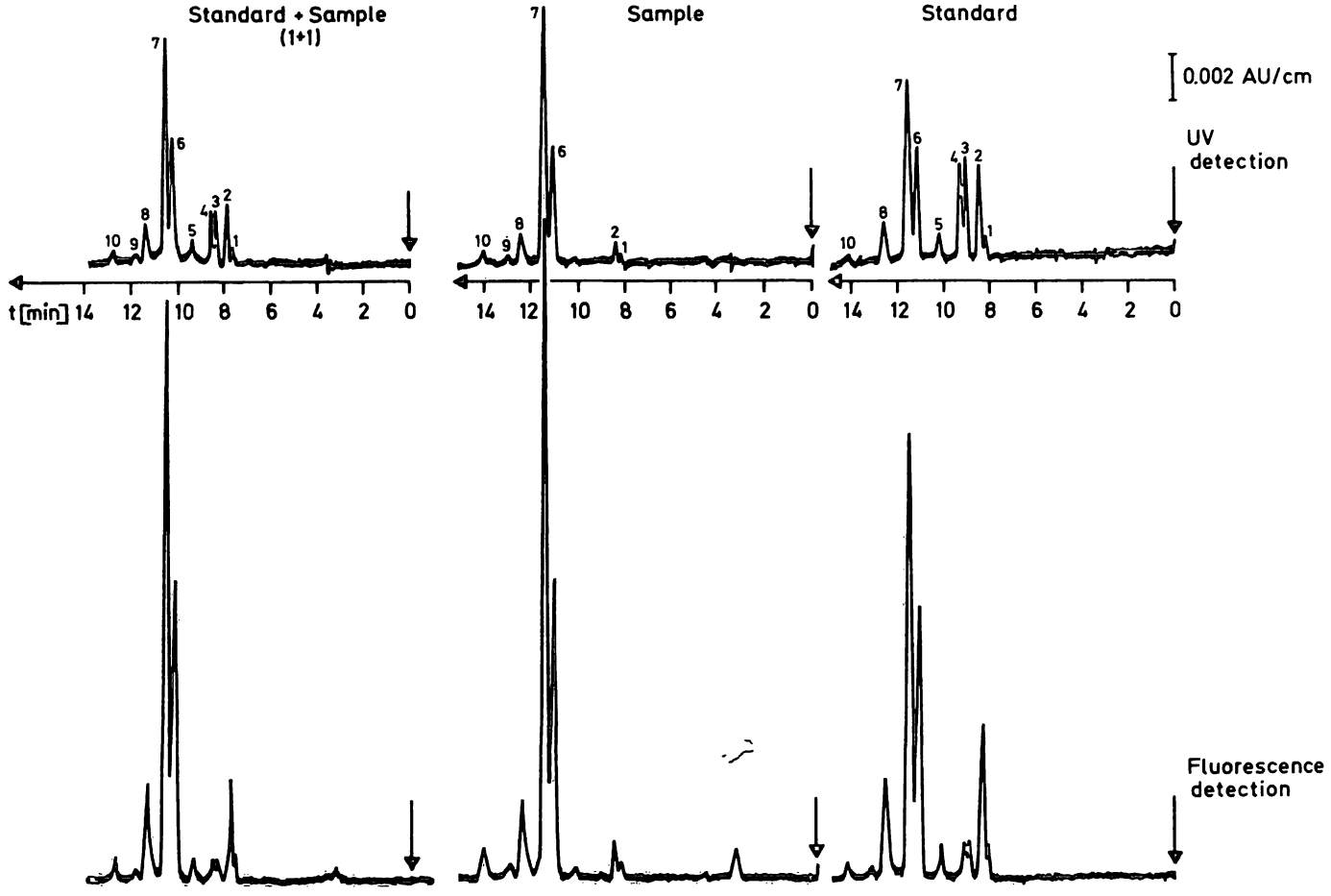

Fig. 2. Chromatogram of a tissue sample and a standard mixture, both containing known concentrations, and a chromatogram of a mixture of "spiked" tissue sample and standard. Equilibration 15 hours. Chromatographic conditions as described. UV-sensitivity 0.04 AUFS, fluorescence sensitivity 10.
Peak identification:
1: 13-*cis* retinyl stearate,
2: 13-*cis* retinyl palmitate,
3: 11-*cis* retinyl stearate,
4: 11-*cis* retinyl palmitate,
5: 9-*cis* retinyl palmitate,
6: all-*trans* retinyl stearate,
7: all-*trans* retinyl palmitate,
8: all-*trans* retinyl oleate,
9: all-*trans* retinyl palmitoleate,
10: all-*trans* retinyl linolate.
AU = Absorbance unit FS = full scale

zation during treatment of the tissues with the extraction solvent. Known concentrations of retinyl ester standards dissolved in the extraction solvent were added to a biological sample. Figure 2 shows the chromatogram of the standard mixture, the chromatogram of the biological sample (testicle), and the chromatogram of standard mixture and sample after addition of the standard mixture (500 µl) to the tissue sample homogenate in a volume equivalent to the extraction volume. Table 5 shows the peak height of the standard, the calculated peak height of the biological sample after addition of the standard, the real peak height of the biological sample measured before and the deviation between calculated values and real measurements.

The adsorption isotherm for retinyl esters was found to be linear up to 3 µg and quasi-linear up to 5 µg. Figure 3 represents a typical separation of the different retinyl esters in a liver sample, using fluorescence- and UV-detection.

Precision and linearity

The lower limit of detection for all-*trans* retinyl palmitate was found to be 0.3 pmol (fluorescence) and 0.8 pmol (UV-detection) with a signal-to-noise ratio of 6:1. The limit of detection is similar for retinyl stearate and oleate and slightly less for palmitoleate and linoleate (due to increased retention times and band broadening). Figure 4 represents the detection of retinyl esters in inner ear tissues and demonstrates the detection limits for small samples. Peak 7, for example, represents a total of 7.5 pmol retinyl palmitate.

Retinyl esters can be accurately measured in samples as small as 50 µg dry weight (in the case of the inner ear), although the recovery is better with samples of 1 mg or more. Linearity and recovery of an internal standard (retinyl acetate 75 µg/l) added to different small samples of a guinea pig liver homogenate are given in table 6. According to tables 4 and 6, it is obvious that the coefficient of variation decreases with

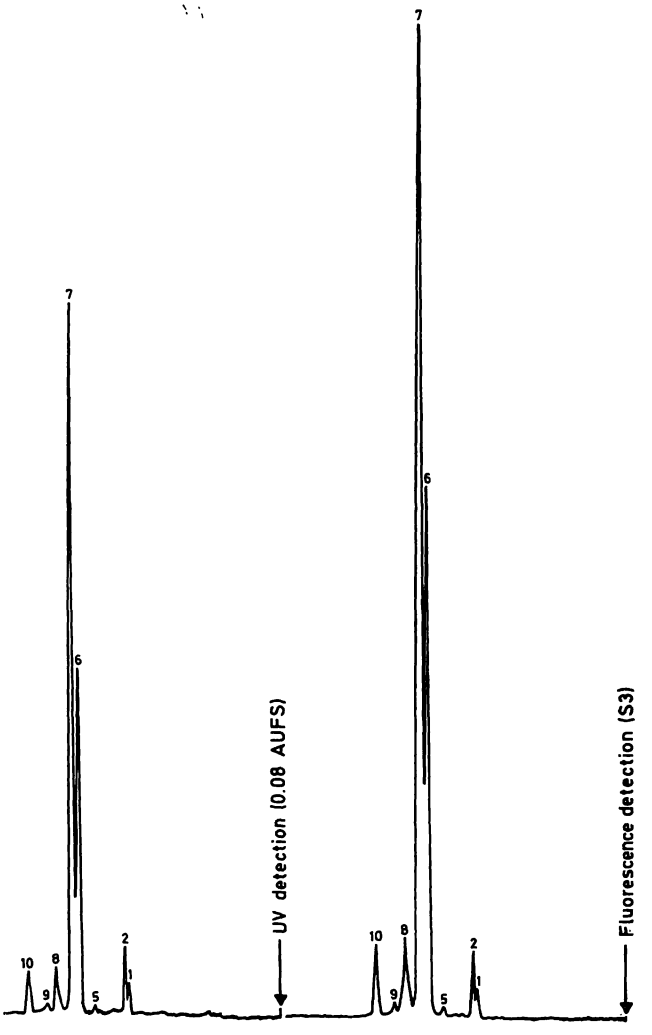

Fig. 3. Separation of different retinyl esters in a liver sample. UV sensitivity 0.08 absorbance units full scale (AUFS), fluorescence sensitivity 3. Peak identification as in figure 2.

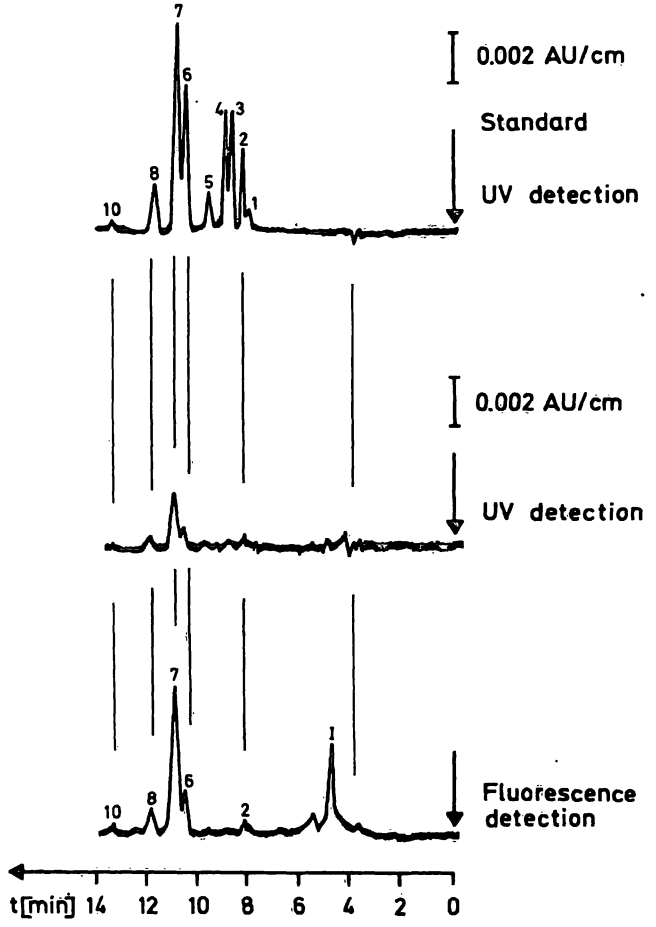

Fig. 4. Chromatogram of retinyl esters in the sensory cells (organ of *Corti*) of the inner ear. Peak identification as in figure 2. UV-sensitivity 0.04 absorbance units full scale (AUFS), fluorescence sensitivity.
AU = absorbance unit

Table 6. Precision and linearity of retinyl palmitate and stearate (ng per sample weight) in small tissue volumes of a powdered liver.

Liver (mg)	Retinyl palmitate	CV %	Retinyl stearate	CV %	Recovery %*
1.0 (6)	158.1 ± 9.3	3.6	31.5 ± 1.8	5.7	101.0
0.5 (3)	121.0 ± 6.7	5.5	16.5 ± 1.2	7.2	94.2
0.25 (5)	68.4 ± 4.9	7.1	6.1 ± 0.7	11.4	96.5
0.1 (5)	30.1 ± 3.4	10.2	4.2 ± 0.6	14.3	89.2

Number of replicates of different weighed samples of a powdered liver in parentheses.
* Recovery based on added retinyl acetate (75 µg/l) as internal standard.

Tab. 7. Absorbance ratios (at 250/325 nm) of pure retinyl ester standards.

		Absorbance ratio		Concentration	
Palmitate	Mean	1.20	n: 9	106.3	
	SD	0.05	CV: 4.2%	1.5	CV: 1.4%
Stearate	Mean	1.43	n: 9	110.3	
	SD	0.15	CV: 10.8	2.3	CV: 2.1%

increasing weight and concentration. For trace analyses with small tissue samples, a quantitative coefficient of variation of about 10% can be accepted (17).

As mentioned above, the accuracy of qualitative and quantitative trace analysis strongly depends on the reproducibility of retention times during the actual separation period. The reproducibility (three replicates) of the retention time of all compounds in a liver sample (fig. 3) ranged from 98.2 to 100.4%. After six to eight separations, a regeneration cycle (1 – 2 hours) is required, due to contamination of the extraction solvent with water, which leads to a slight impairment of separation and alteration of the retention time.

Qualitative analysis

The qualitative analysis was carried out by different methods: Comparison of typical retention times of external standard and sample, recording of fluorescence and/or UV-spectra in the stop flow mode (as mentioned above), and peak purity testing by the absorbance ratio method (16). Table 7 shows the data of the absorbance ratio of a pure standard. Impurities, even in small concentrations, can be detected, due to typical alterations of the ratio. However, "impurities" resulting from vitamin A-derivatives can hardly be separated by this method because the differences in the mixture e. g. palmitate and stearate are not significant under trace analytical conditions. Thus, to detect the co-chromatography of retinyl esters, e. g. of different isomers, we used the detector-quotient method as recommended by *Snyder & Kirkland* (17).

This response ratio method is quite favourable for trace analysis, because the ratio is within a typical range for different retinyl ester isomers as estimated by replicate analyses (tab. 8A). According to *Alvarez* (13) typical co-chromatography of retinyl esters can occur. However, with the response ratio method, it is possible to identify such co-chromatographing compounds from typical alterations of the response ratios (tab. 8B).

Tab. 8A. Mean values of typical detector-quotients, fluorescence: UV, of different isomers of retinyl esters.

	13-*cis*	11-*cis*	9-*cis*	all-*trans*
Mean	1.78	0.374	1.75	2.81
SD	0.07	0.01	0.08	0.07
CV	3.9%	2.8%	4.5%	2.4%
n	15	15	15	21

Tab. 8B. Alteration of detector quotients due to co-chromatography of different retinyl esters.

Standard		Typical quotient range	Co-chromatographing peak	Alteration
11-*cis*	16:0	0.35 – 0.4	13-*cis* 18:1	> 0.4
9-*cis*	18:0	1.65 – 1.85	11-*cis* 18:1	< 1.6
all-*trans*	18:0	2.75 – 2.9	9-*cis* 18:1	< 2.7

Tab. 9. Distribution of retinyl esters in biological samples.

Sample	(n)	Concentration (μg/g dry weight)				
		16:0	18:0	18:1	16:1	18:2
Liver	14	105.3 ± 31.5	45.6 ± 9.2	12.7 ± 2.8	4.8 ± 0.9	16.7 ± 5.5
Kidney	6	1.31 ± 0.65	0.72 ± 0.30	0.11 ± 0.08	0.02 ± 0.01	0.15 ± 0.06
Testicle	7	5.72 ± 4.20	2.88 ± 1.95	0.71 ± 0.43	0.06 ± 0.02	1.22 ± 0.75
Trachea	6	0.18 ± 0.11	0.13 ± 0.07	0.11 ± 0.05	n.d.	0.09 ± 0.04
Inner ear	12	4.8 ± 0.3	1.6 ± 0.2	0.8 ± 0.4	n.d.	0.04 ± 0.1
Tongue	10	1.81 ± 0.68	0.97 ± 0.46	0.28 ± 0.21	0.08 ± 0.06	0.14 ± 0.11

Tissue samples

The distribution of different retinyl esters was determined in different tissues of the guinea pig. The results are shown in table 9. Retinyl palmitate is the main fatty acid ester in all tissues, followed by retinyl stearate, oleate and linoleate. Retinyl palmitoleate shows the smallest concentration in all organs. No *cis* isomer of any retinyl ester could be detected in the different samples. Only in the liver, testicle and kidney did we find small amounts of 13-*cis*- and 9-*cis*-palmitate.

Discussion

Separation of retinyl esters, using various columns and mobile phases has been previously reported by several authors. These methods are not, however, practicable for the trace analysis of different retinyl esters and their geometric isomers in small biological samples.

The methods reported recently are suitable for the detection of retinyl esters in different organs, where either high concentrations of retinyl esters are present, or there is enough tissue to concentrate them by repeated extraction. *Bhat & Lacroix* (18) described a reverse-phase system which allows separation of 9 different naturally occurring retinyl esters in various organs with a limit of detection of 40 pmol, but with retention times of 80 minutes (retinyl stearate). Geometric isomers were not identified. *Furr* et al. (19) described a more sensitive separation of 9 retinyl esters on reverse phase columns with a high resolution, short retention time and good sensitivity (8 pmol retinyl palmitate equivalent to 2.5 ng retinol). However, *cis*-isomers of the different retinyl esters were neither separated nor identified. In a gradient reverse-phase system described earlier (20), retinyl esters were not completely separated and their geometric isomers were not detected. In addition, the high extraction volumes, used in these methods are impracticable for the extraction of small tissue samples (less than 100 μg). *Ross* (14) recently described a reverse-phase gradient system for separation of retinyl esters. The volumes of extraction solvents needed are much too high for trace analysis, especially in the case of small samples. The sensitivity was 74 pmol for individual esters. Furthermore *cis*-isomers were not identified. Thus a method which separates retinyl esters and their geometric isomers under trace analytical conditions was not previously available. Such a method must fulfil special demands of trace analysis, such as extraction in small volumes without derivatisation and evaporation steps, high peak heights with adequate resolution, and separation of *cis-trans* isomers of typical biologically occurring retinyl esters.

It must be remembered that several experimental factors have a marked effect on the sensitivity, accuracy and reproducibility of trace analysis in HPLC. These include the substance, the extraction procedure, the column resolution and sample injection, and the calibration technique.

First, the trace analysis of vitamin A esters, is difficult because these derivatives can undergo rapid changes during analytical handling. Owing to the sensitivity of these compounds to oxygen, light and pH changes, it is necessary to show that the extraction- and separation-procedure is not accompanied by in vitro alterations of the vitamin A esters. Especially when small tissue samples with low concentrations of retinyl esters are involved, it is necessary to avoid additional evaporation or derivatisation steps, because these would lead to an uncontrolled loss of material and rapid alteration of the retinyl esters.

To overcome this problem, we devised a method for the extraction of retinyl esters from small biological samples which allows one-step transfer of the small volume of the extraction solvent to the analytical column, with very brief oxygen contact in the absence of light. In addition, changes in band shape and resolution can be minimized by using the mobile phase to dissolve the sample. However, the extracted retinyl esters are usually separated on a reverse-phase system (18–20), thus making it necessary to adapt the apolar extraction solvents to the more polar mobile phase of

the reverse-phase system. Because we use an adsorption system we are able to transfer the apolar extraction phase to the analytical column without any additional step. Thus the determination of different retinyl esters from small biological samples, as demonstrated here, requires only three steps: freeze-drying, extraction in a solvent similar to the mobile phase, centrifugation and injection on the analytical column. This allows rapid sample preparation and ensures that the retinyl esters will be detected quantitatively without significant in vitro degradation or isomerisation.

The quantitative recovery of the all-*trans* retinyl esters and 13-*cis* retinyl esters from biological samples clearly shows that significant isomerization of retinyl esters does not occur during treatment with the extraction solvent, *n*-hexane (tab. 5). This is in agreement with the findings of *Landers & Olson* (21) who demonstrated that retinyl esters were more stable with respect to isomerization and degradation during extraction and separation with *n*-hexane than other commonly used solvents. In addition, the lower velocity of this mobile phase compared with e. g. acetonitrile- or methanol-containing mobile phases of reverse-phase systems (18—20), results in an increase of peak height (17), which is given preference over the area, since it can hardly be influenced by deviations in flow, noise or baseline drift (22, 23).

Factors, which influence the peak height and therefore the sensitivity and reproducibility, such as temperature deviations and the constancy of solvent composition can be more strictly controlled than flow, noise or baseline drift. Contamination of the solvents with water, which is especially disadvantageous in adsorption chromatography, is avoided, and a constant solvent composition is ensured by a closed recycling system.

The use of this closed recycling system has proved successful in achieving constancy of solvent composition, irrespective of the water content of the mobile phase, resulting in reproducible retention times and a good separation of the retinyl esters. These stable chromatographic conditions are necessary both the quantitative and the qualitative analysis, especially for the application of the detector-quotient method, which allows identification of co-chromoatographing *cis*-isomers of retinyl esters under trace analytic conditions.

The stabilized chromatographic conditions, achieved with the closed recycling system, allow trace analysis of the main biologically occurring retinyl esters and their geometric isomers in small biological samples with high sensitivity, accuracy and reproducibility. It was therefore possible to detect vitamin A storage sites in different tissues such as inner ear, tongue and tracheal mucosa (tab. 9) for the first time.

As demonstrated, the retinyl esters are similar in all tested organs. In all cases retinyl palmitate is the main ester, followed by stearate and oleate. The functional significance of these "vitamin A stores" was recently determined by electrophysiological methods (25) and it is concluded that vitamin A is necessary for hearing acuity, and that the sensitivity is altered when inner ear vitamin A stores are depleted.

Cis-isomers of retinyl esters were usually not detected in biological samples, but in different organs such as liver, testicle and kidney we determined small amounts of 13-*cis*-stearate and -palmitate. It is still unclear whether these isomers are endogenous derivatives or whether they are formed in vitro. However, the fact that in other samples, such as lung, trachea and nasal mucosa, 13-*cis*-derivatives were not detected, in contrast to the tissues discussed above, raises the possibility that 13-*cis* retinyl esters occur in vivo, as shown for 11-*cis* retinyl esters in the retina (11). In addition it should be taken in consideration that up to now geometric isomers of retinyl esters have not so far been determined in biological samples, due to the fact that appropriate methods were not available. Thus our method has a further advantage in that it is possible to detect geometric isomers of retinyl esters in small tissue samples under carefully controlled conditions which, according to our data, avoid in vitro isomerisation of the derivatives, when retinyl ester storing tissues are extracted.

Apart from lung (26) and liver (9), typical storage cells had not previously been demonstrated. However, it can be assumed that even in organs such as the inner ear, storage sites may be present in the form of the well known lipid droplets of the *Hensen* cells near to the sensory cells of the organ of *Corti*.

The determination of traces of retinyl esters and their geometric isomers in small tissue samples enables research laboratories dealing in vitamin A metabolism to test the distribution of observed vitamin A derivatives in special tissues, such as mucous epithelia of the tracheobronchial tract, selected areas of the gut, which can be obtained by fine needle biopsy, or in chylomicron remnants as a parameter for further quantification of these lipid particles. Thus a method is available which allows the determination of retinyl ester stores in humans, and will probably enable the investigation of specific problems, such as the identification of marginal deficiency in general, or in selected tissues.

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